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RESEARCH PAPER

Influence of temperature on measurements of the CO_2 compensation point: differences between the Laisk and O_2-exchange methods

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Abstract

The CO_2 compensation point in the absence of day respiration (Γ*) is a key parameter for modelling leaf CO_2 exchange. Γ* links the kinetics of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) with the stoichiometry of CO_2 released per Rubisco oxygenation from photorespiration (α), two essential components of biochemical models of photosynthesis. There are two main gas-exchange methods for measuring Γ*: (i) the Laisk method, which requires estimates of mesophyll conductance to CO_2 (g_m) and (ii) measurements of O_2 isotope exchange, which assume constant values of α and a fixed stoichiometry between O_2 uptake and Rubisco oxygenation. In this study, the temperature response of Γ* measured using the Laisk and O_2-exchange methods was compared under ambient (25 °C) and elevated (35 °C) temperatures to determine whether both methods yielded similar results. Previously published temperature responses of Γ* estimated with the Laisk and O_2-exchange methods in *Nicotiana tabacum* demonstrated that the Laisk-derived model of Γ* was more sensitive to temperature compared with the O_2-exchange model. Measurements in *Arabidopsis thaliana* indicated that the Laisk and O_2-exchange methods produced similar Γ* at 25 °C; however, Γ* values from O_2 exchange were lower at 35 °C compared with the Laisk method. Compared with a photorespiratory mutant (*pmdh1pmdh2hpr*) with increased α, wild-type (WT) plants had lower Laisk values of Γ* at 25 °C but were not significantly different at 35 °C. These differences between Laisk and O_2 exchange values of Γ* at 35 °C could be explained by temperature sensitivity of α in WT and/or errors in the assumptions of O_2 exchange. The differences between Γ* measured using the Laisk and O_2-exchange method with temperature demonstrate that assumptions used to measure Γ*, and possibly the species-specific validity of these assumptions, need to be considered when modelling the temperature response of photosynthesis.

Key words: CO_2 compensation point, peroxisomes, photorespiration, photorespiratory CO_2 release, photosynthesis, Rubisco oxygenation, temperature.

Introduction

Models of photosynthesis are important tools for predicting the response of plants to climate change. The Farquhar, von Caemmerer, and Berry biochemical model of C_3 photosynthesis was first parameterized to predict photosynthetic rates at 25 °C using the kinetic parameters of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), the enzyme responsible for

Abbreviations: Γ, CO_2 compensation point in the presence of day respiration; Γ*, CO_2 compensation point in the absence of day respiration; α, stoichiometry of CO_2 released per Rubisco oxygenation; A, net CO_2 assimilation rate; A-C_c, net CO_2 assimilation rate as a function of chloroplastic CO_2; C^*, apparent CO_2 compensation point in the absence of day respiration; C_c, chloroplastic CO_2 partial pressure; g_m, mesophyll conductance of CO_2; HPR, hydroxypyruvate reductase; J_{max}, maximum rate of photosynthetic electron transport; K_c, Michaelis–Menten constant for Rubisco carboxylation; K_o, Michaelis–Menten constant for Rubisco oxygenation; O, partial pressure of O_2; PIB, post-illumination burst; PMDH, peroxisomal malate dehydrogenase; R_d, day respiration; Rubisco, ribulose-1,5-bisphosphate carboxylase-oxygenase; $S_{c/o}$, Rubisco specificity of CO_2 over O_2; SE, standard error; v_c, rate of Rubisco carboxylation; V_{cmax}, maximum rate of Rubisco CO_2 carboxylation; v_o, rate of Rubisco oxygenation, WT, wild type.

initiating carbon fixation (Farquhar et al., 1980). This model has proven to be robust in predicting the effects of CO_2 availability on photosynthesis at 25 ºC but has been expanded to account for the temperature response of Rubisco kinetics with mixed success (Bernacchi et al., 2001, 2002, 2003; Sage et al., 2008). For example, the temperature response of the initial slope of the photosynthetic CO_2 response (A-C_i) curve in *Picea mariana* is less than that predicted by the *Nicotiana tabacum* Rubisco kinetic's temperature model (Sage et al., 2008; Way and Sage, 2008). The authors attributed this deviation to a greater deactivation of Rubisco with temperature in *P. mariana* compared with *N. tabacum* or to differences in the temperature response of Rubisco kinetics between these species.

For accurate modelling, it is important to have correct Rubisco kinetics and assumptions concerning the major fluxes of CO_2 and O_2 during photosynthesis. The biochemical model of photosynthesis predicts net leaf CO_2 exchange from the balance of carbon gain through Rubisco carboxylation with carbon loss through day respiration (R_d) and photorespiration. Photorespiration releases CO_2 at a given stoichiometry of CO_2 per oxygenation (α), which is assumed to remain constant at 0.5 based on current understanding of photorespiratory biochemistry (Reumann and Weber, 2006). In C_3 plants, photorespiration releases carbon at approximately 25% the rate of gross CO_2 fixation, reducing the quantum efficiency of photosynthesis (von Caemmerer and Farquhar, 1981; Sharkey, 1988). Therefore, the CO_2 compensation point in the absence of day respiration (Γ^*), which quantifies photorespiratory loss of CO_2 and the kinetic properties of Rubisco, is an essential term in models of photosynthesis (see Equations 2 and 4 below).

Γ^* can be measured either biochemically through *in vitro* assays or *in vivo* using gas-exchange methods. Generally, *in vivo* Γ^* is measured with the so-called 'Laisk method' as the intersection of A-C_i curves measured at multiple subsaturating light intensities (Γ^*_L) (Laisk, 1977). The original method described by Laisk did not take into account mesophyll conductance of CO_2 (g_m) (Equation 3) to adjust the intercellular CO_2 partial pressure (C_i) to the CO_2 partial pressure at the site of Rubisco (C_c); however, several recent publications have reviewed the importance of including g_m in estimates of Γ^*_L and gas-exchange generally (Warren, 2008; Furbank et al., 2009). Alternatively, mass spectrometer measurements of leaf O_2 isotope exchange can also be used as an *in vivo* estimate of Γ^* (Γ^*_O). This method does not require estimates of g_m but does require assumptions related to leaf O_2 exchange and α (see Equations 4, 7, and 8) (Ruuska et al., 2000; Bernacchi et al., 2002). O_2 exchange is typically measured by placing a leaf disk in a sealed cuvette in an $^{18}O_2$ atmosphere attached to a mass spectrometer via a membrane inlet (Canvin et al., 1980; Beckmann et al., 2009). The exchange of O_2 in and out of the leaf is measured by following the uptake of $^{18}O_2$ and evolution of the natural abundance of $^{16}O_2$ from water splitting during photosynthesis (see Equations 7 and 8). The Γ^*_O calculations assume that α is constant at 0.5, O_2 consumption from day respiration is the same as in the dark, and rates of photoreduction of O_2 to water (the Mehler reaction) are negligible (Canvin et al., 1980; Badger, 1985). These assumptions appeared valid at 25 ºC when compared with independent measurements of gas exchange and Rubisco kinetics (Ruuska et al., 2000), but their accuracy as temperature increases has not been widely characterized (Badger et al., 2000).

Unfortunately, measurements of α are inherently difficult because they require determining the rate of CO_2 release from photorespiration and the rate of Rubisco oxygenation (v_o) while Rubisco carboxylation (v_c) and CO_2 release from R_d continue in the light. However, at 25 °C the post-illumination burst (PIB) and $^{12}CO_2$ release following a saturating $^{13}CO_2$ injection both scale with photorespiratory CO_2 release, providing an estimate of the CO_2 component of α (Doehlert et al., 1979; Delfine et al., 1999; Loreto et al., 2001; Cousins et al., 2008, 2011). Additionally, v_o can be estimated using isotopic exchange of $^{18}O_2$ and $^{16}O_2$, but these measurements are subject to the assumptions of O_2 exchange outlined previously and discussed in the theory section below (Canvin et al., 1980; Badger, 1985; Cousins et al., 2008, 2011). Recently, measurements of $^{12}CO_2$ release and $^{18}O_2$ and $^{16}O_2$ exchange indicated an increase in α in *Arabidopsis thaliana* lacking both isoforms of peroxisomal malate dehydrogenase (*pmdh1pmdh2*) and peroxisomal hydroxypyruvate reductase (*hpr*) (Cousins et al., 2008, 2011).

Despite the importance of Γ^* to gas-exchange models and the value of understanding the temperature response of photosynthesis, to our knowledge there are no published comparisons of Γ^*_L and Γ^*_O at ambient and elevated temperatures. Such a comparison would help determine whether the two methods give consistent results and identify which assumptions may need re-evaluating at elevated temperatures. Therefore, this study examined the temperature response of Γ^*_L and Γ^*_O measured in *N. tabacum* (Bernacchi et al., 2001, 2002). Additionally, the temperature and O_2 response of Γ^*_L and Γ^*_O were measured in *A. thaliana* wild-type (WT) and *pmdh1pmdh2hpr* plants. These data were used to explore the potential physiological explanations for differences between the two measurements of Γ^*, including increases in α and changes in O_2 exchange with temperature.

Theory

The rate of net assimilation of CO_2 (A) can be modelled by subtracting CO_2 released by photorespiration and mitochondrial respiration from Rubisco carboxylation rates:

$$A = v_c - \alpha v_o - R_d \quad (1)$$

where R_d is the rate of day respiration (Farquhar et al., 1980). Additionally, the Farquhar, von Caemmerer, and Berry biochemical model describes Rubisco-limited photosynthesis as:

$$A = V_{cmax}\left(\frac{C_c - \Gamma^*}{C_c + K_C\left(1 + O/K_O\right)}\right) - R_d \quad (2)$$

where V_{cmax}, K_c, and K_o represent the maximum rate of v_c and Michalis–Menten constants for reactions with CO_2 and O_2,

respectively (von Caemmerer, 2000). C_c can be calculated from intercellular CO_2 partial pressure (C_i) using g_m according to:

$$C_c = C_i - \frac{A}{g_m} \quad (3)$$

Γ^*, the CO_2 compensation point in the absence of day respiration is described by the Rubisco specificity for CO_2 over O_2 ($S_{c/o}$), partial pressure of O_2 (O) and α as:

$$\Gamma^* = \frac{\alpha O}{S_{c/o}} = \frac{\alpha \Gamma v_o}{v_c} \quad (4)$$

Changes in Γ^* affect estimates of net assimilation and, as indicated in Equation 4, are directly proportional to O and α. The CO_2 compensation point in the presence of R_d (Γ) is expressed as:

$$\Gamma = \frac{\Gamma^* + K_c\left(1 + O/K_o\right)R_d/V_{cmax}}{1 - R_d/V_{cmax}} \quad (5)$$

and is measured as the CO_2 partial pressure where A is zero.

Photosynthesis at higher CO_2 partial pressures is not Rubisco limited (Equation 2) but is usually limited by the ability of the Calvin—Benson cycle to regenerate intermediates for carbon fixation due to insufficient production of NADPH. Under these conditions, photosynthesis is dependent on the maximum rate of electron transport (J_{max}) and energy demand of photosynthesis and photorespiration according to:

$$A = \frac{\left(C_c - \Gamma^*\right)J_{max}}{4C_c + 8\Gamma^*} - R_d \quad (6)$$

The Laisk method of measuring Γ^* (Γ^*_L) is limited by the ability to measure g_m accurately to convert measured values of C_i to C_c (Equation 3), whereas Γ^* measured on the mass spectrometer (Γ^*_O) relies on estimates of v_o and v_c and assumes α=0.5 (Equation 4) (Ruuska et al., 2000; Bernacchi et al., 2002). In this method, v_o is determined assuming that rates of O_2 uptake in the dark are equal to uptake by mitochondrial respiration in the light. Additionally, the total O_2 uptake by Rubisco is determined assuming 1 mole of O_2 is consumed from the atmosphere during oxidation of glycolate in the peroxisome for every two oxygenations of Rubisco (Badger, 1985). Therefore, the rate of v_o is:

$$v_o = 2/3\left({}^{18}O_2 \text{ uptake in the light} - {}^{18}O_2 \text{ uptake in the dark}\right) \quad (7)$$

Assuming that all electrons from water splitting reduce NADPH for v_c and v_o (Badger, 1985; Ruuska et al., 2000), v_c can be determined by:

$$v_c = {}^{16}O_2 \text{ evolution in the light} - v_o \quad (8)$$

Subsequently, Γ^*_O can then be calculated from v_c and v_o (Equation 4). Both the Laisk and the O_2-exchange method rely on measurements of the net exchange of CO_2 and O_2, respectively, to determine Γ^* assuming that CO_2 and O_2 are exchanged primarily through reactions of photosynthesis, photorespiration, and R_d. However, there are several other carboxylases and decarboxylations within plant cells, including phosphoenolpyruvate carboxylase and carbamoyl phosphate synthetase, that could mask the true Γ^* with unaccounted fluxes (Raven and Farquhar, 1990). Whilst these additional fluxes are important physiologically, their rates are typically a tenth to a one-thousandth the rate of CO_2 flux through Rubisco and have a negligible impact on calculations of Γ^*

Materials and methods

Growth conditions

WT *A. thaliana* Columbia accession and mutant *pmdh1pmdh2hpr* (Pracharoenwattana et al., 2007) were grown in a climate-controlled cabinet (Econair Ecological Chambers, Winnipeg, Canada) under a photosynthetic flux density of 300 µmol m^{-2} s^{-1} and 2000 µbar CO_2 to minimize the phenotype of *pmdh1pmdh2hpr1* (Pracharoenwattana et al., 2007). Day/night cycles were 11/13 h and 23/18 °C. Seeds were cold stratified for 3 d and germinated on sterile agar plates supplemented with MS medium (Plant Media, Dublin, OH, USA) and 1% sucrose. Following cold stratification, plates were placed in the growth chamber for 1 week and the seedlings were then transferred to soil for an additional 3 weeks and fertilized weekly with Peters 20-20-20 (J.R. Peters, Allentown, PA, USA). The youngest fully expanded leaves of 31–40-d-old plants were used for gas-exchange measurements.

Laisk CO_2 compensation points

The Laisk method (Laisk, 1977) was used to measure the apparent compensation point (C^*) in WT and *pmdh1pmdh2hpr* plants under different O at 25 and 35 °C. Different O_2 partial pressures (92, 184, and 368 mbar O_2) were generated using O_2 and N_2 mixed with calibrated mass flow controllers (model GFC17; Aalborg, Orangeburg, NY, USA). A-C_i curves were measured on a leaf fully enclosed in a 2 cm^2 measuring head (6400–40 Leaf Chamber Fluorometer; Li-Cor Biosciences, Lincoln, NE, USA) at subsaturating light intensities using a Li-Cor 6400 XT (Li-Cor Biosciences) and the x and y coordinates of these points were used to determine R_d (y coordinate) and C^* (x coordinate). CO_2 diffusion through the gasket was corrected according to the manufacturer's instructions (Li-cor 6400XT manual version 6). The CO_2 compensation point in the absence of day respiration (Γ^*) was subsequently calculated from C^* by accounting for mesophyll conductance (g_m) and R_d according to $\Gamma^* = C^* + R_d/g_m$, with g_m equal to 0.2 and 0.35 mol CO_2 m^{-2} bar^{-1} at 25 and 35 °C, respectively. The value of 0.2 mol CO_2 m^{-2} bar^{-1} at 25 °C was the average of several *A. thaliana* ecotypes measured under various conditions (Tazoe et al., 2011) and this value becomes 0.35 mol CO_2 m^{-2} bar^{-1} at 35 °C according to the temperature-response model of (Bernacchi et al., 2002).

Mass spectrometric measurements

Rates of v_c and v_o were determined from measurements of $^{18}O_2$ consumption and $^{16}O_2$ evolution according to Equations 7 and 8. $^{18}O_2$ consumption in the light and dark and $^{16}O_2$ evolution in the light was measured as described previously (Canvin et al., 1980;

Ruuska et al., 2000; Cousins et al., 2008). Briefly, a leaf disc was placed in a temperature-controlled closed cuvette system connected to a mass spectrometer (Delta V; Thermo Scientific) via a temperature-controlled membrane inlet. The cuvette was flushed with N_2 gas, injected with $^{18}O_2$ gas to obtain a given O_2 partial pressure, and sealed. $^{18}O_2$ consumption and $^{16}O_2$ evolution rates were monitored during dark and light periods. The PIB was determined from the maximum transient rate of CO_2 release in the dark following a 10 min period of illumination. The $^{12}CO_2$ release was determined in the light from the maximum rate of $^{12}CO_2$ released following a saturating injection of $^{13}CO_2$ (Delfine et al., 1999; Loreto et al., 2001; Cousins et al., 2008, 2011). $^{13}CO_2$ was generated from acid-released $^{13}CO_2$ from 98% $^{13}CO_2$/sodium hydrogen carbonate (Sigma Aldrich, St Louis MO, USA). Γ^* was calculated according to Equation 4.

Parameterization and temperature-response modelling

A-C_c curves were measured using a Li-Cor 6400 assuming a g_m of 0.2 and 0.35 mol CO_2 m^{-2} bar^{-1} at 25 and 35 °C, respectively (Bernacchi et al., 2002; Tazoe et al., 2011). Measurements were made under saturating illumination (photosynthetic flux density of 1200 µmol m^{-2} s^{-1}) and vapour pressure deficits below 15 mbar at 25 °C and 25 mbar at 35 °C. The A-C_c curves were fitted to the leaf photosynthesis model (Farquhar et al., 1980) using *in vivo N. tabacum* Rubisco constants at 25 and 35 °C to determine the effects of changes in Γ^* on V_{cmax} and J_{max} (Bernacchi et al., 2001, 2002). The Γ^* temperature and O_2 response was modelled by first changing the scaling constant of Bernacchi et al. (2001, 2002) so that the function gave values of Γ^* that matched WT *A. thaliana* at 25 °C. The heat of activation determined previously in *N. tabacum* Bernacchi et al. (2001, 2002) was used to model values at 35 °C.

Statistics

A four-way analysis of variance (ANOVA; Table 1) was used to determine the influence of genotype, temperature, measurement method, and O_2 levels on Γ^* using Statistix 9 (Analytical Software, Tallahassee, FL, USA). A three-way ANOVA (Table 2) was used to determine the influence of genotype, temperature, and O_2 levels on measurements of v_o, $^{12}CO_2$ release per v_o and PIB per v_o using Statistix 9. A two-way ANOVA was used to determine the significance in measured and modelled V_{cmax} and J_{max} values (Table 3) using R (R Foundation for Statistical Computing, Vienna Austria, http://www.R-project.org). Significance was assumed to be P <0.05.

Results

Comparison of Γ^*_L and Γ^*_O from Bernacchi et al. (2001, 2002)

The response of Γ^*_L to temperature was modelled as described by Bernacchi et al. (2001) using the Laisk method to estimate the temperature response assuming an infinite mesophyll conductance (g_m) (Fig. 1, dotted line). This response was then corrected for g_m using the values reported by Bernacchi et al. (2002), the temperature response of R_d from Bernacchi et al. (2001), and Equation 3 (Fig. 1, solid line). The dashed line in Fig. 1 represents the temperature response of Γ^*_O according to Bernacchi et al. (2002), which was determined from O_2 exchange using a membrane inlet mass spectrometer assuming a constant stoichiometric release of CO_2 per oxygenation of 0.5. The 25 °C Γ^*_L values were greater than Γ^*_O regardless of g_m and were more responsive to temperature.

Effects of Γ^*_L and Γ^*_O on modelling of photosynthetic CO_2-response curves under elevated temperatures

In WT *A. thaliana*, the photosynthetic parameters V_{cmax} and J_{max} were calculated (Farquhar et al., 1980; von Caemmerer, 2000) from the net CO_2 assimilation rates as a function of CO_2 partial pressures at 25 and 35 °C assuming Γ^*_L, Γ^*_O, and Rubisco kinetics from Bernacchi et al. (2001, 2002, 2003) (Fig. 2 and Table 1). V_{cmax} was not significantly different when Γ^*_L or Γ^*_O was used at 25 °C (41.3 ± 1.5 µmol m^{-2} s^{-1} for Γ^*_L and 38.1 ± 0.5 µmol m^{-2} s^{-1} for Γ^*_O). However, at 35 °C, V_{cmax} was significantly different depending on whether Γ^*_L (85.9 ± 5.1 µmol m^{-2} s^{-1}) or Γ^*_O (70.8 ± 2.1µmol m^{-2} s^{-1}) was used for the calculation. Additionally, at 35 °C, the modelled temperature response of V_{cmax} was significantly different from the measured values using Γ^*_O (Bernacchi et al., 2002) but not Γ^*_L (Bernacchi et al., 2001). However, the calculated values of J_{max} were not significantly different when using either Γ^*_O or Γ^*_L. Additional key gas-exchange parameters, including net CO_2 assimilation, intercellular CO_2 concentration, stomatal conductance to H_2O, and H_2O transpiration rates, are presented in Supplementary Table S1 (at *JXB* online).

Table 1. Effects of using the Laisk or O_2-exchange methods to estimate Γ^* on modelling CO_2 assimilation curves under elevated temperature. Maximum rate of Rubisco carboxylation (V_{cmax}) and electron transport (J_{max}) at 25 and 35 °C calculated using standard biochemical models of leaf photosynthesis (von Caemmerer, 2000) with Γ^*_L or Γ^*_O from Bernacchi et al. (2001) or Bernacchi et al. (2002). The modelled V_{cmax} and J_{max} at 35 °C were scaled from 25 °C measurements using the temperature-response functions of Bernacchi et al. (2001, 2002). Results are shown as means ±SE of five leaves from separate plants. Statistical analysis was conducted using a one-way ANOVA; different superscript letters indicate significant differences between α assumptions and temperatures at P <0.05.

Temperature	Assumed Γ^*	V_{cmax} (µmol m^{-2} s^{-1})		J_{max} (µmol m^{-2} s^{-1})	
		Measured	Modelled	Measured	Modelled
25°C	Γ^*_L	41.3 ± 1.5^a	–	87.6 ± 3.1^a	–
	Γ^*_O	38.1 ± 0.5^a	–	84.6 ± 2.7^a	–
35°C	Γ^*_L	85.9 ± 5.1^c	96.5 ± 3.4^c	107.9 ± 3.1^b	163.2 ± 6.2^c
	Γ^*_O	70.8 ± 2.1^b	89.0 ± 1.2^c	97.0 ± 3.0^{ab}	148.9 ± 4.8^c

Table 2. Results of ANOVA comparing the Laisk and O_2-exchange methods of measuring Γ* at various oxygen partial pressures and temperatures in WT and *pmdh1pmdh2hpr A. thaliana*. Method refers to the measurement technique of Γ* with measurements of O_2 exchange on the mass spectrometer indicated as Mass to avoid confusion with the *O* effect and the mutant *pmdh1pmdh2hpr* referred to as 3X for convenience. *O* is indicated as the partial pressure in mbar (92_{O2}, 184_{O2}, and 368_{O2}) and temperature is in °C (25_T, 35_T). Asterisks indicate a significant interaction according to ANOVA (P <0.05) and different superscript letters denote significant differences according to a Tukey's post-hoc test (P <0.05). Results are shown as the means ±SE of three to six leaves from separate plants.

Parameter	Factor	$F_{ndf, ddf}$	Interactions
Γ*	Genotype	$72.2_{1,87}$*	
	O_2	$397.3_{2, 87}$*	
	Temp	$84.7_{1, 87}$*	
	Method	$0.6_{1, 87}$	
	Genotype, O_2	$6.1_{2, 87}$*	
	Temp, Method	$4.5_{1, 87}$*	
	Genotype, Method	$4.9_{1, 87}$*	
	Genotype, O_2, Temp	$1.6_{5, 87}$	
	Genotype, Temp, Method	$15.4_{1, 87}$*	$3X35_TLaisk^{ab}$, $3X35_TMass^{a}$, $3X25_TLaisk^{bc}$, $3X25_TMass^{bc}$, $WT35_TLaisk^{ab}$, $WT35_TMass^{cd}$, $WT25_TLaisk^{de}$, $WT25_TMass^{e}$
	O_2, Temp, Method	$3.8_{4, 87}$*	$368_{O2}35_TLaisk^{a}$, $368_{O2}35_TMass^{a}$, $368_{O2}25_TLaisk^{b}$, $368_{O2}25_TMass^{b}$, $184_{O2}35_TLaisk^{b}$, $184_{O2}35_TLaisk^{bc}$, $184_{O2}25_TLaisk^{cd}$, $184_{O2}25_TLaisk^{de}$, $92_{O2}35_TLaisk^{e}$, $92_{O2}35_TLaisk^{f}$, $92_{O2}35_TLaisk^{f}$, $92_{O2}35_TLaisk^{f}$
	Genotype, O_2, Temp, Method	$2.0_{4, 87}$	–

CO_2 compensation point in the absence of day respiration under elevated temperature

Γ^*_L and Γ^*_O were measured and modelled in *A. thaliana* WT and *pmdh1pmdh2hpr* plants at 25 and 35 °C in response to various *O* (Fig. 3). WT values of Γ^*_L and Γ^*_O increased linearly with *O* at both 25 °C (r^2=1.0000 for Γ^*_L and r^2=0.9997 for Γ^*_O) and 35 °C (r^2=0.9982 for Γ^*_L and r^2=0.9854 for Γ^*_O) with a significantly higher Γ^*_L compared with Γ^*_O at 35 °C regardless of *O* (Fig. 3A, C and Table 2). There was also a linear response of Γ^*_L and Γ^*_O in the *pmdh1pmdh2hpr* plants to *O* at 25 °C (r^2=0.9999 for Γ^*_L and r^2=0.9423 for Γ^*_O) and 35 °C (r^2=0.9979 for Γ^*_L and r^2=0.9773 for Γ^*_O) (Fig. 3B, D); however, there was no significant difference between Γ^*_L and Γ^*_O at either temperature (Table 2). The WT Γ^*_L and Γ^*_O was lower than *pmdh1pmdh2hpr* at 25 °C regardless of *O* (Table 2). However, at 35 °C, there was no difference in Γ^*_L between the two genotypes, but Γ^*_O was significantly higher in *pmdh1pmdh2hpr* compared with WT at all *O* (Table 2).

As the model was fitted to WT values of Γ^*_L and Γ^*_O at 25 °C for Bernacchi et al. (2001, 2002), there was good agreement between the measured and modelled values at each *O* for the WT. However, at 35 °C, Γ^*_L was slightly underestimated and Γ^*_O was slightly overestimated by Bernacchi et al. (2001). The modelled Γ* for the *pmdh1pmdh2hpr* plants was adjusted to a higher α of 0.8 (Equation 4). At 25 °C in the *pmdh1pmdh2hpr* plants, the modelled values fitted Γ^*_L at all *O* and Γ^*_O at 92 and 184 mbar *O* (Fig. 3B). However, at 35 °C, Γ^*_L was underestimated by the model of Bernacchi et al. (2001) and Γ^*_O was overestimated by the model of Bernacchi et al. (2002) (Fig. 3D).

Rates of Rubisco oxygenation and carboxylation from measurements of O_0 and of CO_2 isotope exchange

A membrane inlet mass spectrometer was used to measure rates of CO_2 and O_2 isotope exchange in WT and *pmdh1-pmdh2hpr* plants in response to temperature and *O*. From these measurements, the PIB, the release of $^{12}CO_2$ in a saturating $^{13}CO_2$ background, and the rate of Rubisco oxygenation (v_o) were determined. At 25 and 35 °C, there was a significant response of v_o to *O* for both genotypes (Fig. 4 and Table 3). However, v_o did not respond to temperature in WT plants but was significantly different between 25 and 35°C in the *pmdh1pmdh2hpr* plants. In WT plants, the *PIB*:v_o ratio was significantly lower at 25 °C compared with that at 35 °C, regardless of O_2 level, and was significantly lower in WT compared with *pmdh1pmdh2hpr* plants at 25 but not at 35°C across all O_2 levels (Fig. 5 and Table 3). In both genotypes, there was no significant response of PIB:v_o to O_2 at 25 °C, but at 35 °C, the PIB:v_o ratio was higher at 92 mbar compared with at 184 and 368 mbar O_2. The $^{12}CO_2$:v_o ratio responded significantly to O_2, regardless of temperature and genotype, but decreased with temperature in the *pmdh1pmdh2hpr* plants but not in the WT plants. At 25 °C, the $^{12}CO_2$:v_o ratio was greater in the *pmdh1pmdh2hpr* plants compared with the WT plants, but there was no difference between genotypes at 35 °C. In summary, at 25 °C the

Table 3. Results of two-way ANOVA on mass spectrometric measures of CO_2 release during photorespiration in WT and *pmdh1pmdh2hpr A. thaliana*. ANOVA analysis between rates of Rubisco oxygenation (v_o), PIB:v_o, and $^{12}CO_2$ release following a saturating injection of $^{13}CO_2$ ($^{12}CO_2$):v_o as measured on leaf punches with a membrane inlet mass spectrometer. The mutant *pmdh1pmdh2hpr* is referred to as 3X for convenience, *O* is indicated by the partial pressure in mbar (92_{O2}, 184_{O2}, and 368_{O2}) and temperature is in °C (25_T, 35_T). Asterisks indicate a significant interaction according to ANOVA (P <0.05) and different superscript letters denote significant differences according to a Tukey's post-hoc test (P <0.05). Results are shown as the means ±SE of three to six leaves from separate plants.

Parameter	Factor	$F_{ndf, ddf}$	Interactions
v_o	Genotype	$37.7_{1,72}$*	
	Temp	$22.4_{1,72}$*	
	O_2	$148.1_{2,72}$*	
	Genotype, O_2	$14.9_{2,72}$*	$WT92_{O2}^{c}$, $WT184_{O2}^{b}$, $WT368_{O2}^{a}$, $3X92_{O2}^{c}$, $3X184_{O2}^{b}$, $3X368_{O2}^{b}$
	Genotype, Temp	$37.7_{1,72}$*	$WT25_T^{a}$, $WT35_T^{a}$, $3X25_T^{b}$, $3X35_T^{a}$
	Temp, O_2	$4.5_{1,72}$*	$25_T92_{O2}^{d}$, $25_T184_{O2}^{c}$, $25_T368_{O2}^{b}$, $35_T92_{O2}^{d}$, $35_T184_{O2}^{b}$, $35_T368_{O2}^{a}$
	Genotype, O_2, Temp	$2.4_{2,72}$	–
PIB/v_o	Genotype	$54.3_{1,72}$*	
	Temp	$2.2_{1,72}$	
	O_2	$7.8_{2,72}$*	
	Genotype, O_2	$5.9_{2,72}$*	$WT92_{O2}^{a}$, $WT184_{O2}^{b}$, $WT368_{O2}^{b}$, $3X92_{O2}^{a}$, $3X184_{O2}^{a}$, $3X368_{O2}^{a}$
	Genotype, Temp	$21.8_{1,72}$*	$WT25_T^{c}$, $WT35_T^{b}$, $3X25_T^{a}$, $3X35_T^{ab}$
	Temp, O_2	$17.2_{1,72}$*	$25_T92_{O2}^{b}$, $25_T184_{O2}^{b}$, $25_T368_{O2}^{ab}$, $35_T92_{O2}^{a}$, $35_T184_{O2}^{b}$, $35_T368_{O2}^{b}$
	Genotype, O_2, Temp	$2.6_{2,72}$	–
$^{12}CO_2/v_o$	Genotype	$4.8_{1,72}$*	
	Temp	$0.1_{1,72}$	
	O_2	$8.9_{2,72}$*	
	Genotype, O_2	$2.4_{2,72}$	
	Genotype, Temp	$15.9_{2,72}$*	$WT25_T^{b}$, $WT35_T^{ab}$, $3X25_T^{a}$, $3X35_T^{b}$
	Temp, O_2	$0.3_{2,72}$	–
	Genotype, O_2, Temp	$2.4_{2,72}$	–

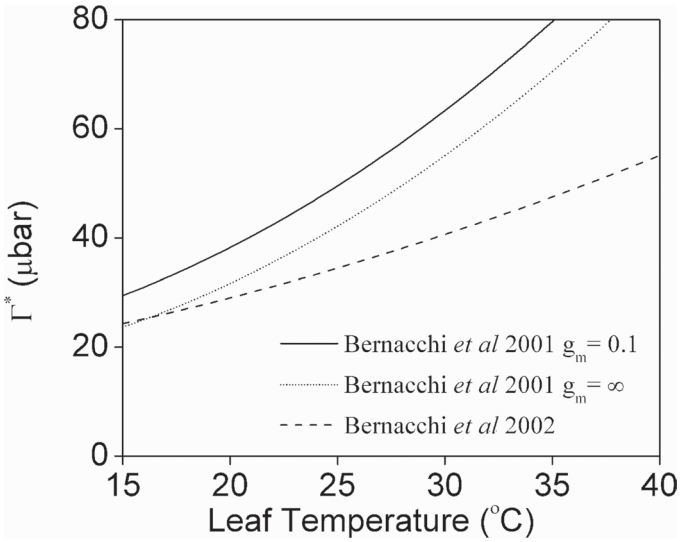

Fig. 1. Modelled response of the CO_2 compensation point in the absence of day respiration (Γ*) to temperature and O_2. The graph shows the temperature response of Γ* from Bernacchi et al. (2001) with a correction for mesophyll conductance (g_m) (solid line) and with an infinite g_m (dotted line). The dashed line represents the response according to Bernacchi et al. (2002), which was determined assuming a stiochiometric release of CO_2 per oxygenation of 0.5.

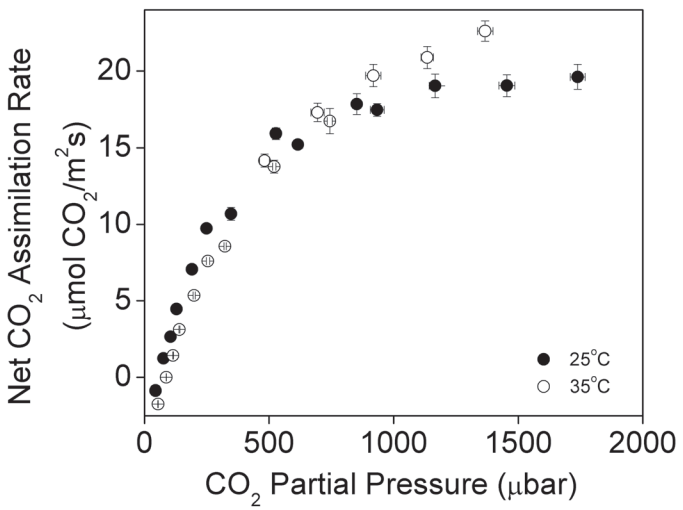

Fig. 2. The CO_2 response of photosynthesis at 25 °C (closed circles) and 35 °C (open circles) as measured by a Li-Cor 6400. Chloroplastic CO_2 partial pressure (C_c) was determined from previously published mesophyll conductance values (with a mesophyll conductance (g_m) of 0.2 and 0.35 mmol m^{-2} s^{-1} bar^{-1} at 25 °C (Tazoe et al., 2011) and 35 °C, respectively. Results are shown as means ±standard error (SE) of five leaves from separate plants.

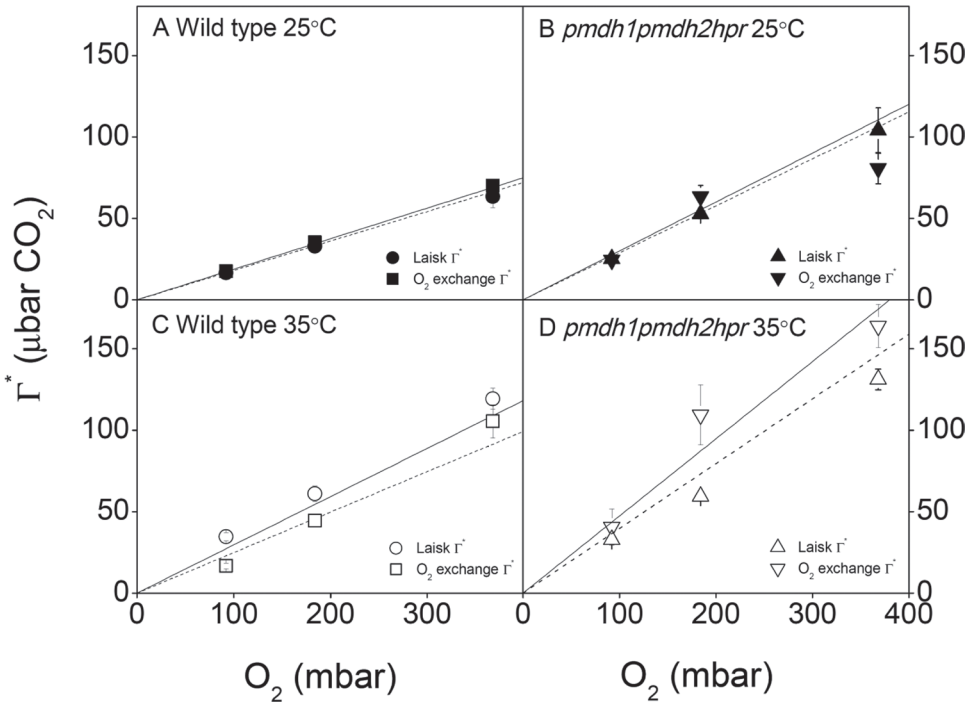

Fig. 3. The CO_2 compensation point in the absence of day respiration (Γ*) in WT (A, C) and *pmdh1pmdh2hpr* (B, D) *A. thaliana* plants under various O_2 partial pressures at 25 °C (closed symbols; A, B) and 35 °C (open symbols; C, D) measured using the Laisk method for WT (circles) and *pmdh1pmdh2hpr* (upward triangles) plants. Measurements of Γ* using O_2 exchange are also shown for WT (squares) and *pmdh1pmdh2hpr* (downward triangles) plants. Solid lines represent predicted Γ* values from Bernacchi et al. (2001) with a mesophyll conductance (g_m) of 0.2 and 0.35 mmol m^{-2} s^{-1} bar^{-1} at 25 and 35 °C, respectively. Dotted lines show the results from Bernacchi et al. (2002) with α=0.8 for the *pmdh1pmdh2hpr* plants. Results are shown as means ±SE of three to six leaves from separate plants. Laisk data (25 °C) from WT and *pmdh1pmdh2hpr* plants were also presented in Cousins et al. (2011).

PIB:v_o and $^{12}CO_2$:v_o ratios were higher in the *pmdh1pmdh2hpr* plants compared with the WT plants, but at 35°C, they were not significantly different between genotypes, regardless of *O*. Additionally, *PIB*:v_o was significantly higher in the WT plants at 35 °C compared with 25 °C but did not significantly respond to temperature in the *pmdh1pmdh2hpr* plants. In the WT plants, *PIB*:v_o was different at 92 mbar compared with at 184 and 368 mbar O_2 but not in the *pmdh1pmdh2hpr* plants.

Discussion

Effects of R_d and g_m on measurements of Γ using the Laisk and O_2-exchange methods*

Bernacchi et al. (2001) measured the temperature response of Γ* in *N. tabacum* using the Laisk method (Γ^*_L) (Laisk, 1977) to develop a temperature response model of Γ*. Measurements of Γ^*_L require no assumptions about the

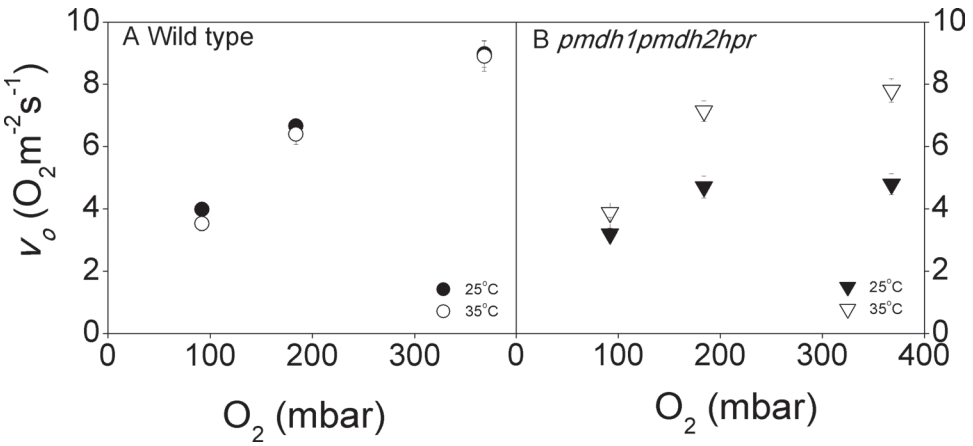

Fig. 4. Rates of Rubisco oxygenation (v_o) at the CO_2 compensation point estimated from measurements of $^{18}O_2$ and $^{16}O_2$ exchange as described in Materials and methods. Measurements were made at 92, 184, and 368 mbar O_2 at 25 and 35°C for both WT (A) and *pmdh1pmdh2hpr* (B) *A. thaliana* plants. Results are shown as means ±SE of three to six leaves from separate plants.

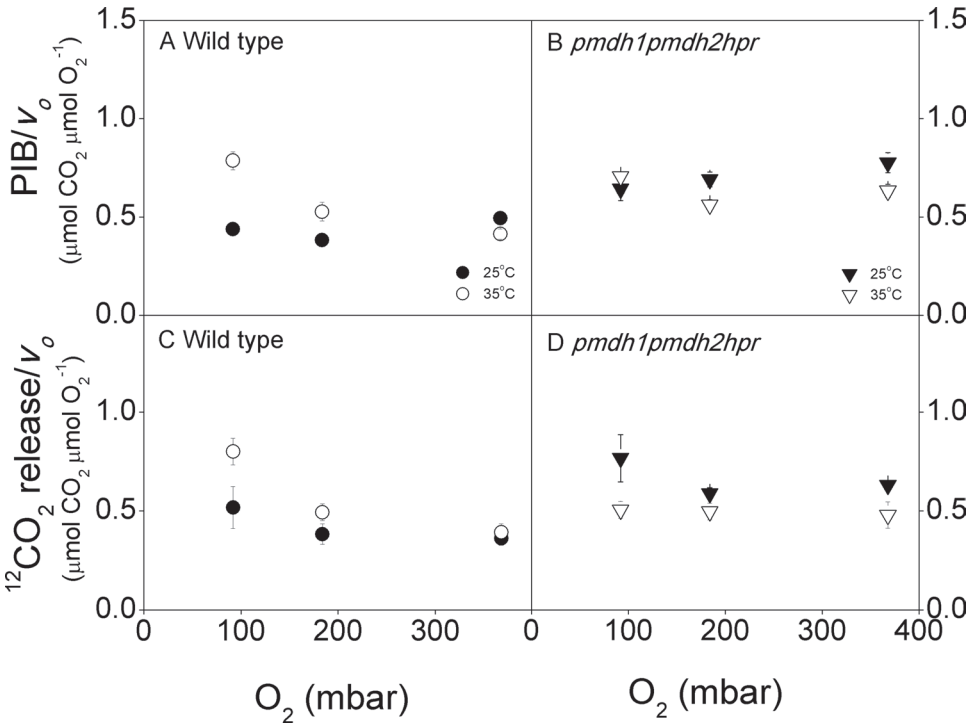

Fig. 5. PIB per v_o (A, B) and $^{12}CO_2$ release per v_o (C, D) at various O_2 partial pressures in WT (A, C) and *pmdh1pmdh2hpr* (B, D) *A. thaliana* at 25 and 35 °C measured from CO_2 and isotopic O_2 with the membrane inlet mass spectrometer. Results are shown as means ±SE of three to six leaves from separate plants.

photorespiratory stoichiometry of CO_2 released per oxygenation (α) or leaf O_2 exchange but must be corrected for the difference between C_i and C_c with values of mesophyll conductance to CO_2 (g_m) (Equation 3). Additionally, the temperature response of Γ^* was measured using O_2 exchange at Γ (Γ^*_O) (Bernacchi et al., 2002), which does not require values of g_m (see below), but assumes that: (i) α is equal to 0.5 (Equation 4), (ii) O_2 is consumed only by photorespiration and Rubisco oxygenation, (iii) rates of O_2 consumption by R_d are the same as respiration in the dark, and (iv) all electrons passed to NADPH drive either photosynthesis or photorespiration (Equations 4, 7, and 8) (Badger, 1985). Given that all these assumptions are correct, then Γ^*_L should equal Γ^*_O. However, a direct comparison of these two methods of estimating Γ^* has not been conducted, particularly in response to temperature.

As noted in several publications, it is important to account for g_m to measure Γ^*_L accurately (von Caemmerer et al., 1994; von Caemmerer, 2000; Ethier and Livingston, 2004; Furbank et al., 2009). This is because Γ^*_L is determined from the intercept of several A-C_i curves measured under subsaturating light conditions. The x value of this intercept represents C_i at Γ^*, and the y value of A is negative and represents rates of R_d (Laisk, 1977). To estimate Γ^*_L, the values of C_i must be corrected for g_m to obtain an accurate C_c (Equation 3) (Ethier and Livingston, 2004; Furbank et al., 2009). As Γ^*_L is determined when A is negative, Γ^*_L before accounting for g_m is lower than after correcting for g_m. The Γ^*_L values reported by Bernacchi et al. (2001) were uncorrected for g_m, meaning that they are lower than the g_m-corrected value would be. Therefore, to accurately describe Γ^* the model of Bernacchi et al. (2001) must be corrected for the temperature response of g_m. Alternatively, at Γ, there is no net photosynthesis and the ratio of A:g_m approaches zero regardless of g_m value (Equation 3). Therefore, because A is zero at Γ, measurements of C_i are equal to C_c. Under these conditions, stomatal conductance is similarly negligible and C_c can be determined from measured CO_2 partial pressure inside the sealed cuvette without correcting for g_m. Consequently, measurements of Γ^*_O are not sensitive to errors in g_m (Equation 4). This latter approach was used by Bernacchi et al. (2002) to measure Γ^* independently of assumptions of g_m. To compare these two models of Γ^* at 25 °C, the values of Bernacchi et al. (2001) for Γ^*_L were corrected using g_m according to Bernacchi et al. (2002). At 25 °C, the g_m-corrected modelled values of Γ^*_L were higher than the modelled Γ^*_O (Fig. 1) and differences between Γ^*_L and Γ^*_O increased with temperature, highlighting the greater temperature sensitivity of Γ^*_L compared with Γ^*_O. The difference between Γ^*_L and Γ^*_O is not explained by potential errors in assumptions of g_m because the difference is significant when g_m is assumed to be infinite (no restriction to CO_2 diffusion and C_i=C_c) and the discrepancy increases as g_m decreases (Fig. 1). Similarly, any assumed value of R_d (from zero to infinity) also increases the discrepancy between the values of Γ^*_L from Bernacchi et al. (2001) and the values of Γ^*_O from Bernacchi et al. (2002) (Equation 3). In summary, the temperature response and absolute values of Γ^*_L are higher than Γ^*_O even when corrected for g_m and regardless of R_d. Both of these estimates of Γ^* are used to determine the maximum rate of Rubisco carboxylation (V_{cmax}) and the maximum

rate of electron transport (J_{max}) from gas-exchange measurements of A-C_i curves (von Caemmerer, 2000). Additionally, the temperature response of Γ^* is essential for modelling the response of these parameters and photosynthesis to changes in leaf temperatures. Therefore, it is important to determine how the difference in temperature response of Γ^* between Bernacchi et al. (2001) and Bernacchi et al. (2002) influences estimates of V_{cmax} and J_{max} derived from A-C_c measurements. To test this, V_{cmax} and J_{max} were determined from A-C_c curves measured at 25 and 35 °C in *A. thaliana* with Γ^* from the two Bernacchi et al. (2001, 2002) temperature-response models.

Sensitivity of V_{cmax} and J_{max} to Γ^*

At temperatures above 25 °C, previous publications have attributed lower V_{cmax} estimated from leaf gas-exchange measurements compared with modelled values as changes in the Rubisco activation state (Sage et al., 2008). However, some of this difference could also be explained by errors in Γ^* and its modelled temperature response. For example, using the g_m-corrected Γ^*_L from Bernacchi et al, (2001) to compare measured and modelled V_{cmax} values from *A. thaliana* A-C_c curves, there was no significant difference at 35 °C (Table 1). However, if Γ^*_O from Bernacchi et al. (2002) was used to calculate V_{cmax}, then the measured values were significantly lower than the modelled V_{cmax} (Table I). This could be interpreted as deactivation of Rubisco using Γ^*_O but not with Γ^*_L. This difference between V_{cmax} calculated using Γ^*_L versus Γ^*_O highlights the importance of determining which method is most appropriate for modelling photosynthesis at different temperatures, as well as understanding which assumptions within the two models are valid in response to changing temperatures.

It is possible that the differences in Γ^*_L (Bernacchi et al., 2001) versus Γ^*_O (Bernacchi et al., 2002) are dependent on the differences in Rubisco content between genotypes used for each study. For example, Rubisco antisense plants were used by Bernacchi et al. (2001) to measure the temperature response of Γ^*_L. These plants have lowered photosynthetic rates compared with WT plants, which may have introduced errors into measuring the intercept of A-C_i curves at low CO_2 partial pressures (Hudson et al., 1992). Indeed, the 25 °C value of Γ^*_L of 41.9 μbar CO_2 from antisense plants measured by Bernacchi et al. (2001) is higher than other reports of Γ^*_L measured in both WT *N. tabacum* and other C_3 plants. For example, Γ^*_L values at 25 °C typically range between 36.7 and 40.8 μbar CO_2 (Brooks and Farquhar, 1985; von Caemmerer et al., 1994; Laisk and Loreto, 1996).

Additionally, differences between Γ^*_L and Γ^*_O could be driven by errors in the assumptions used to parameterize each method. As previously discussed, Γ^*_L must be corrected for g_m; however, including corrections for g_m increases the difference between Γ^*_L and Γ^*_O. Alternatively, there are several assumptions used in determining Γ^*_O with unknown temperature responses. For example, measurements of Γ^*_O assume that α is constant at 0.5 (Equation 4). Additionally, Γ^*_O relies on measurements of v_o and v_c, which require assumptions relating O_2 exchange to Rubisco reactions (discussed below). Therefore, to test these assumptions at 25 and 35°C, measurements of Γ^*_L and Γ^*_O were made in *A. thaliana* WT and the photorespiratory mutant (*pmdh1pmdh2hpr*), previously characterized as having an increased α, to determine which parameters contribute to the discrepancies between Γ^*_L and Γ^*_O.

Differences in Γ^*_L and Γ^*_O in WT A. thaliana

Measurements of Γ^*_L and Γ^*_O in *A. thaliana* were used to confirm and characterize the differences between the two methods of measuring Γ^* presented by Bernacchi et al. (2001, 2002) (Fig. 3). In *A. thaliana*, there was no significant difference between Γ^*_L and Γ^*_O in WT plants at 25 °C (Table 2). This is different from what was observed previously in *N. tabacum* where Γ^*_L determined by Bernacchi et al. (2001) was higher than Γ^*_O from Bernacchi et al. (2002) at 25 °C (Fig. 1). The different response of Γ^*_L and Γ^*_O at 25 °C between *A. thaliana* and *N. tabacum* could be the result of the different genotypes used in each study. As mentioned before, Bernacchi et al. (2001) used Rubisco small-subunit antisense plants, whilst Bernacchi et al. (2002) measured WT *N. tabacum;* however, in the current study, WT *A. thaliana* plants were used for both estimates of Γ^*.

The close agreement of Γ^*_L and Γ^*_O in WT *A. thaliana* at 25 °C at a variety of O values provides strong support that the independent assumptions of both methods are valid at 25 °C in this species. However, at 35 °C, Γ^*_L and Γ^*_O in *A. thaliana* were significantly different across all O (Table 2 and Fig. 3), confirming a similar increased temperature response of Γ^*_L over Γ^*_O as suggested by the comparison of data from Bernacchi et al. (2001, 2002). As discussed previously, larger values of g_m increase Γ^*_L estimated from measured C_i values (Equation 3); however, regardless of the g_m values used (0.10 to infinity), Γ^*_L was always larger than Γ^*_O (data not shown). Therefore, errors in g_m do not explain the differences between Γ^*_L and Γ^*_O at 35 °C. These findings in *A. thaliana* confirm differences in Γ^*_L and Γ^*_O above 25 °C, although the difference is less than reported in *N. tabacum* (Bernacchi et al., 2001, 2002). To determine whether the different temperature responses of Γ^*_L and Γ^*_O could be explain by changes in α the photorespiratory mutant *pmdh1pmdh2hpr* was compared with WT at both 25 and 35°C.

Response of α to temperature

Biochemical models of photosynthesis and measurements of Γ^*_O (Equation 4) typically assume α=0.5 under all conditions. However, there are several recent publications demonstrating changes in α when the traditional photorespiratory pathway is disrupted through genetic manipulation. For example, the photorespiratory mutants *pmdh1pmdh2*, *hpr*, and *pmdh1-pmdh2hpr* had lower net photosynthetic rates under photorespiratory conditions, higher Γ and higher Γ^*_L than WT plants (Cousins et al., 2008, 2011). Additionally, measurements of CO_2 and O_2 isotope gas exchange in the *pmdh1pmdh2* and *hpr* plants at 25 °C confirmed that Γ and Γ^*_L were higher due to an increase in α (Cousins et al., 2008, 2011). Similar to

previously published work on *hpr* and *pmdh1pmdh2* plants, the photorespiratory mutant *pmdh1pmdh2hpr* in this study had higher Γ^*_L and CO_2 release per v_o compared with WT plants, indicating an increase in α in the *pmdh1pmdh2hpr* plants at 25 °C (Fig. 5 and Table 3, discussed below). The Γ^*_O value in the *pmdh1pmdh2hpr* plants was modelled with α=0.8 instead of α=0.5, a stoichiometry that also modelled Γ and Γ^*_L in this and previous studies with photorespiratory mutants with increased α (Equation 4 and Fig. 3) (Cousins et al., 2008, 2011). This suggests that misestimates of α can lead to inaccurate calculations of Γ^*_O.

Measurements of Γ^*_L, which do not require assumptions of α, in WT plants were significantly lower than in *pmdh1-pmdh2hpr* plants at 25 °C; however, at 35 °C, the values were not significantly different between genotypes across all *O* (Table 2). It is expected that the $S_{c/o}$ of Rubisco is conserved between WT and *pmdh1pmdh2hpr* plants at a given temperature (Jordan and Ogren, 1984); therefore, differences in Γ^*_L at 25 °C could be attributed to α (Equation 4). However, at 35 °C, the values of Γ^*_L were the same between WT and *pmdh1pmdh2hpr* plants, suggesting that α may increase with temperature in WT *A. thaliana* to a stoichiometry similar to that in *pmdh1pmdh2hpr* plants. In WT plants, an increase in α could also explain why Γ^*_L and Γ^*_O were the same at 25 °C but Γ^*_O was lower than Γ^*_L at 35 °C when assuming α=0.5. The linear response of Γ^*_L to O_2 at 35 °C indicated that the increase in α would be constant at a given temperature, regardless of *O* (Equation 4 and Fig. 3).

Two putative reactions of photorespiratory intermediates within the peroxisome could explain increases in α. Specifically, excess glyoxylate and hydroxypyruvate could react with H_2O_2 releasing CO_2, formate, and glycolate with or without an enzyme catalyst (Elstner and Heupel, 1973; Halliwell, 1974). Indeed, this reaction is hypothesized to be a major source of formate in leaves (Igamberdiev et al., 1999). Formate can be further decarboxylated in the peroxisome (Halliwell and Butt, 1974) or oxidized to CO_2 by formate dehydrogenase in the mitochondria (Hourton-Cabassa et al., 1998), whilst glycolate could re-enter the photorespiratory pathway. These reactions would result in additional CO_2 release per Rubisco oxygenation and divert carbon from the Calvin–Benson cycle and the regeneration of ribulose-1,5-bisphosphate.

It has also been hypothesized that, in WT plants, similar increases in α occur under elevated temperatures due to an increase in glycolate oxidase activity relative to catalase within the peroxisomes (Grodzinski and Butt, 1977). Additionally, in isolated peroxisomes and mitochondria, an increase in H_2O_2 can react with glyoxylate and hydroxypyruvate leading to an increase release of CO_2 (Grodzinski and Butt, 1977; Grodzinski, 1978; Hanson and Peterson, 1985). Furthermore, overexpression of catalase in *N. tabacum* reduced the levels of H_2O_2 and lowered Γ as temperature increased compared with WT plants (Brisson et al., 1998). These data suggest that α could increase from non-catalysed decarboxylation reactions with H_2O_2, decreasing the efficiency of phosphoglycolate recycling but not completely disrupting the photorespiratory pathway. Therefore, measurements of labelled CO_2 and O_2 isotope exchange as described by Cousins et al. (2008, 2011) were used to determine the influence of temperature on α and to probe some of the assumptions of O_2 exchange used to measure Γ^*_O at 35 °C.

Rates of Rubisco oxygenation in A. thaliana WT and pmdh1pmdh2hpr plants

Rates of v_o and v_c are determined by the relative availability of O_2 and CO_2, Rubisco kinetics, and the activation state of Rubisco (Salvucci and Crafts-Brandner, 2004; von Caemmerer et al., 2004; Sage et al., 2008). It has been shown that Rubisco deactivates under high temperatures, decreasing both v_c and v_o (Kobza and Edwards, 1987; Feller et al., 1998). Deactivation of Rubisco at 35 °C could explain the insensitivity of v_o to temperature across all O_2 treatments in WT *A. thaliana*. However, in contrast to the WT, v_o increased with temperature in *pmdh1pmdh2hpr* plants but was constant at 184 and 368 mbar O_2 at 35 °C. This is paradoxical given the apparent decrease in $^{12}CO_2$ release per v_o in *pmdh1pmdh2hpr* plants under higher Rubisco oxygenation conditions when perturbations to photorespiration would be more severe (Table 3 and Fig. 5). However, this could be explained by errors in measuring v_o and/or photorespiratory CO_2 release (discussed below).

Alternatively, the increase in v_o with temperature in *pmdh1pmdh2hpr* but not in WT plants could be attributed to changes in O_2 exchange by alternative oxidations of photorespiratory intermediates traditionally not described as part of the photorespiratory pathway. For example, measurements of v_o would decrease if the 2:3 ratio of v_o to net O_2 uptake used in Equation 7 decreased due to additional oxygenation of photorespiratory intermediates. This would subsequently increase the ratios of PIB and $^{12}CO_2$ release per v_o. These reactions could also explain the apparent discrepancies seen in v_o and CO_2 release per v_o at 35 °C in the *pmdh1pmdh2hpr* plants (Fig. 5). If similar increases in α occur in WT plants under elevated temperature due to non-enzymatic or enzymatic reactions, measurements of v_o and CO_2 release per v_o would also be affected.

CO_2 release per Rubisco oxygenation reaction

To measure α accurately, both the flux of CO_2 from photorespiration and the corresponding rates of v_o must be determined. The CO_2 released from photorespiration cannot be measured directly; however, the combined flux from photorespiration and R_d can be estimated from the PIB and by the rate of $^{12}CO_2$ evolution following a saturating injection of $^{13}CO_2$ on an illuminated leaf (Cousins et al., 2008, 2011). As discussed previously, the photorespiratory mutant *pmdh1pmdh2hpr* had a higher PIB and $^{12}CO_2$ release per v_o at 25 °C compared with WT plants across *O*, suggesting an increased α in the *pmdh1p-mdh2hpr* plants (Fig. 5 and Table 3). However, at 35°C, the PIB per v_o was not significantly different between WT and *pmdh1pmdh2hpr* plants (Fig. 5 and Table 3). Curiously, PIB per v_o was significantly higher at the lowest *O* compared with the other *O* levels in both WT and *pmdh1pmdh2hpr* plants. This increase in CO_2 release per v_o at the lowest *O* was not

expected based on the linear relationship between Γ^*_L and O (Fig. 3).

The discrepancy between Γ^*_L and Γ^*_O and the downward trend in PIB and $^{12}CO_2$ release per v_o in response to O seen in the WT plants might also be explained by errors in two major assumptions of O_2 exchange: (i) the O_2 uptake from day respiration is equal to rates of dark respiration and (ii) the rates of Mehler reaction are negligible. It is generally accepted that rates of respiration in the light (R_d) are less than rates of respiration in the dark (Villar et al., 1994; Lambers and Ribas-Carbo, 2005) and that R_d may respond to changes in rates of photorespiration (Tcherkez et al., 2008). The Laisk measurements of Γ^*_L can estimate the CO_2 release from R_d, which could be used in place of uptake of $^{18}O_2$ in the dark in estimates of v_o assuming a stoichiometry of CO_2 evolution to O_2 uptake during respiration (Equation 7). However, when R_d was used to calculate v_o instead of the measured dark rates of O_2 consumption, there was no change in the trends of PIB and $^{12}CO_2$ release per v_o, as presented in Fig. 5, when the stoichiometry of CO_2 evolution to O_2 uptake was held constant regardless of the value (comparison not shown). Therefore, there would have to be changes in the stoichiometry of CO_2 evolution to O_2 uptake to explain the changes in Fig. 5.

In addition to differences in dark versus light respiration, higher rates of the Mehler reaction at 35 °C could introduce errors in the calculated rates of v_o due to the consumption of O_2 independent of photosynthesis and photorespiration (Ort and Baker, 2002). This would lead to overestimations of v_o (Equation 7) and underestimations of PIB and $^{12}CO_2$ release per v_o. The rates of Mehler would have to range from 10% of O_2 evolution at 92 mbar to 60% at 368 mbar to maintain a constant PIB and $^{12}CO_2$ release per v_o with O (calculations not shown). However, at 25 °C, the rates of Mehler in C_3 plants are reported to range from 0 to 30% of photosynthetic electron transport at 25 °C (Asada, 1999; Badger et al., 2000; Ruuska et al., 2000; Driever and Baker, 2011), but the temperature dependence and O_2 response of these reactions are not well known for *A. thaliana*. Measurements of O_2 exchange under various conditions in *N. tabacum* found that v_o explained O_2 consumption under low and elevated temperatures, suggesting that the Mehler rate does not increase with temperature (Badger et al., 2000). Therefore, the temperature response of the Mehler reactions in *A. thaliana* would have to be significantly different compared with *N. tabacum* to explain the downward trend in Fig. 5.

Finally, the downward trend in PIB and $^{12}CO_2$ release per v_o in response to O seen in the WT plants at 35 °C could be explained if the CO_2 released from photorespiration does not scale with PIB and $^{12}CO_2$ release at elevated temperatures across O. In this situation, PIB and $^{12}CO_2$ release would no longer be proportional to the CO_2 released from photorespiration at 35 °C in response to O. This would lead to a decrease in the ratio PIB and $^{12}CO_2$ release per v_o as O increases that does not correspond to changes in α. The observation that PIB saturates with increasing O at 25 °C and with temperature supports this suggestion (Doehlert et al., 1979). Therefore, at 35 °C, the discrepancy between a constant α described by the linear response of Γ^*_L and the decreasing trend in PIB and $^{12}CO_2$ release per v_o as O increases could be explained by a saturating response of PIB and $^{12}CO_2$ release to photorespiratory rates. Unfortunately, there is insufficient evidence to determine whether the downward trend in PIB and $^{12}CO_2$ release per v_o with O is the result of unaccounted rates of the Mehler reaction, changes in differences between dark and light respiration rates, or saturation of CO_2 released from photorespiration as measured by PIB and $^{12}CO_2$ release. Each of these could individually or collectively affect estimates of PIB and $^{12}CO_2$ release per v_o in response to O.

Conclusion

The data presented here demonstrate differences in temperature-response models of Γ^* from *N. tabacum* between the Laisk and O_2-exchange methods. These differences were large enough to impact both measured and modelled values of V_{cmax} and J_{max}. Differences in Γ^* determined from the Laisk and O_2-exchange method were also seen in *A. thaliana* at 35 °C. The difference in estimates of Γ^* were probably due to errors in assumptions used in O_2-exchange calculations at elevated temperature. The extent of these errors and the species-specific differences in these assumptions should be considered when modelling the temperature response of photosynthesis with Γ^* values derived from O_2 exchange.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Gas-exchange parameters from CO_2-response curves measured at 25 and 35 °C.

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